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We sought to determine the critical threshold level of c-*erb*B-2 overexpression that is required to transform human mammary epithelial cells and to assess the importance of cooperative interactions between $p185^{erbB-2}$ and *erb*B-3 in the neoplastic transformation of breast cancer cells with c-*erb*B-2 gene amplification. We have now successfully produced cell populations derived from MCF-10A non-neoplastic human mammary epithelial cells that overexpress c-*erb*B-2 at very high levels truly comparable to that seen in breast cancer cells with c-*erb*B-2 gene amplification. While the original clones of genetically engineered MCF-10A*erb*B-2 cells overexpressed c-*erb*B-2 at only moderate levels and were not tumorigenic in nude mice, cells selected for high-level c-*erb*B-2 overexpression with growth factor independence in culture showed a high level of constitutive PI 3-kinase activity and a highly transformed phenotype both in culture and *in vivo*. In addition, key observations were made during the course of these studies which provide important information concerning the survival and growth of cells in the absence of growth factors that occurs as a function of the level of c-*erb*B-2 gene overexpression. Finally, we have recently constructed a bicistronic retroviral expression vector containing a dominant negative form of c-*erb*B-3 that specifically inhibits $p185^{erbB-2}$/*erb*B-3 activation, and this has now allowed us to begin to directly test the relative importance of $p185^{erbB-2}$/*erb*B-3 heterodimer function in cells in response to HRG and in various breast cancer cell lines with c-*erb*B-2 gene amplification.

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Stephen P. Ethier, Ph.D.

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INTRODUCTION

Our data now strongly implicates phosphatidylinositol (PI) 3-kinase-mediated signal transduction in heregulin (HRG)-induced mitogenesis, as well as the transformed growth of breast cancer cells with c-*erb*B-2 gene amplification. Our previous work also showed that increasingly elevated levels of c-*erb*B-2 overexpression in breast cancer cell lines are associated with increased constitutive activation of $p185^{erbB-2}$, *erb*B-3 and PI 3-kinase, as well as progressive growth factor independence in culture. Other previous reports had suggested that experimentally elevated c-*erb*B-2 gene expression in non-neoplastic human mammary epithelial cells was sufficient to convert non-neoplastic cell lines to a neoplastic phenotype when injected into nude mice. However, it was also earlier reported that c-*erb*B-2 overexpression alone was not sufficient to fully transform MCF-10A non-neoplastic human mammary epithelial cells. But the MCF-10A*erb*B-2 cell clones that were derived in those studies only showed moderate levels of c-*erb*B-2 gene expression relative to tumor cell lines containing c-*erb*B-2 gene amplification. The minimum level of c-*erb*B-2 gene overexpression that may be required for full transformation of mammary epithelial cells (which, unlike NIH3T3 cells, routinely co-express c-*erb*B-3) has not yet been precisely determined. In addition, none of the above studies with transfected mammary cells have precisely quantified the signal transduction events which underlie these phenomena in experimentally generated c-*erb*B-2-overexpressing human mammary epithelial cells. Therefore, we have completed a series of experiments to address these questions by producing MCF-10A cells that overexpress variously elevated levels of c-*erb*B-2 truly comparable to that seen in breast cancer cell lines with c-*erb*B-2 gene amplification (objective 1). Using these cell lines, we have also completed our goals to determine the critical threshold level of c-*erb*B-2 overexpression that is required to constitutively activate key signaling pathways, induce growth factor independence and effectively transform MCF-10A cells (objective 3). We also sought to assess the importance of the cooperative interactions that occur between $p185^{erbB-2}$ and *erb*B-3 receptor tyrosine kinases during the autonomous growth and neoplastic transformation of breast cancer cells with c-*erb*B-2 gene amplification. While no direct ligand for $p185^{erbB-2}$ has yet been cloned, it is now clear that $p185^{erbB-2}$ is capable of heterodimerization with the other *erb*B kinases, *erb*B-1, *erb*B -3 and *erb*B-4. These $p185^{erbB-2}$-containing heterodimers form the highest affinity binding sites for their respective ligands, epidermal growth factor (EGF) for *erb*B-1 and HRG for *erb*B-3 and *erb*B-4. While c-*erb*B-2 is amplified in 28% of primary breast carcinomas *in vivo*, amplification of c-*erb*B-3 or c-*erb*B-4 was not seen in various studies. However, our own work and others have now shown that heterodimer interactions between $p185^{erbB-2}$ and *erb*B-3 are constitutively activated in breast cancer cells with c-*erb*B-2 gene amplification and co-transfection of c-*erb*B-3 with c-*erb*B-2 greatly augments the transforming capability of c-*erb*B-2 in NIH3T3 cells. The $p185^{erbB-2}$/*erb*B-3 heterodimer complexes are especially potent in activating the PI 3-kinase and other signal transduction pathways in cells stimulated with HRG or in breast cancer cells that constitutively activate $p185^{erbB-2}$/*erb*B-3 due to c-*erb*B-2 gene amplification. By constitutively activating key signal transduction pathways to a level that is effective for transformation, tumor cells escape the normal controls on cell cycle regulation. We are particularly interested in how high the level of c-*erb*B-2 gene expression must be to constitutively activate key signal transduction pathways to a critical level that fully transforms human mammary epithelial cells, as well as how the cooperative effects of $p185^{erbB-2}$/*erb*B-3 heterodimer function affects this critical threshold in different cell types. Therefore, we have also constructed cell lines that express a dominant negative form of *erb*B-3 in which most of the cytoplasmic region was removed in order to inhibit $p185^{erbB-2}$/*erb*B-3 function in various cell lines (objective 2), and to study the effects of dominant negative *erb*B-3 on HRG responsiveness and the transformed growth of breast cancer cells with c-*erb*B-2 gene amplification (objective 4).

BODY

In order to accomplish objectives 1 and 3 of the grant, we first derived cell lines from MCF-10A*erb*B-2 cells that overexpress variously elevated levels of c-*erb*B-2 by using Flow Cytometry and other selection strategies (see previous progress reports). While earlier attempts to derive c-*erb*B-2-overexpressing cell lines were not successful, Flow Cytometry selection of MCF-10A*erb*B-2 cell populations with antibodies directed against cell-surface $p185^{erbB-2}$ was quite effective in deriving a population of cells, called MCF-10A*erb*B-2sh cells, that show much higher levels of c-*erb*B-2 overexpression truly comparable to that seen in breast cancer cells with c-*erb*B-2 gene amplification. Additionally, long-term growth factor deprivation resulted in a population of cells, called MCF-10A*erb*B-2shH cells, with increased levels of $p185^{erbB-2}$ above that seen with Flow Cytometry selection alone. Immunocytochemistry of $p185^{erbB-2}$ levels in the variously selected cell populations also indicated that the selection of growth factor-independent phenotypes by growth factor deprivation leads to a more homogenous population comprised of the highest level c-*erb*B-2-overexpressing cells. While MCF-10A and MCF-10A*erb*B-2 cells die off in the absence of all growth factors in culture, many MCF-10A*erb*B-2sh cells survived after the withdrawal of growth factors under serum-free conditions in culture. This then allowed us to culture these cells for an extended period in the complete absence of growth factors under high cell density culture conditions. After confluent cultures were left a month in the absence of all growth factors, these cells began to develop foci-like structures and these cultures were then passaged at a low split ratio in the absence of any growth factors. As mentioned above, these MCF-10A*erb*B-2shH cells selected in the absence of growth factors showed a more homogenous high-level c-*erb*B-2 overexpression than did the original Flow Cytometry-selected MCF-10A*erb*B-2sh cell population. This suggested that the selection of these c-*erb*B-2-overexpressing cells in the absence of growth factors resulted in the preferential survival of the highest-level overexpressing cells within the original cell population.

The levels of activated $p185^{erbB-2}$ in both Flow Cytometry-selected cell populations, MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells, were considerably elevated over that seen in the original MCF-10A*erb*B-2 cell population as measured in anti-phosphotyrosine Western blots. Western blotting for p85 of PI 3-kinase in anti-phosphotyrosine immunoprecipitates was then done to measure the levels of activated PI 3-kinase in cells under growth factor-free conditions in the different cell lines in culture. As in the 21MT tumor cell lines with c-*erb*B-2 gene amplification, MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells (but not the MCF-10A*erb*B-2 parental cell population) showed constitutive activation of PI 3 kinase, being highest in the MCF-10A*erb*B-2shH cells. Thus, increased levels of PI 3-kinase constitutive activation (i.e. in the complete absence of exogenous growth factors) are associated with the increased levels of $p185^{erbB-2}$.

Dramatic alterations in cell morphology were also observed for the MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cell lines in culture (see previous progress reports). Therefore, we used these MCF-10A*erb*B-2-derived cell lines for further in-depth study of the effects of progressively elevated c-*erb*B-2 gene overexpression on growth factor independence and their transformed phenotype in culture as well as their tumorigenicity *in vivo*. The MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cell lines were found to grow well in the complete absence of insulin-like growth factor (IGF) in serum-free culture and MCF-10A*erb*B-2shH cells proliferated slowly in the absence of EGF in standard 10 day growth assays. MCF-10A*erb*B-2sh and MCF-10A*erb*B-2shH cells also showed much greater responsiveness to the mitogenic effects of HRG both in monolayer and soft agarose culture. Further experiments were carried out to test for the transformed phenotype of MCF-10A*erb*B-2shH cells both in culture and *in vivo*. MCF-10A*erb*B-2shH cells were found to form foci in monolayer culture, and to form large colonies in soft agarose culture

with high efficiency. As reported previously, the original MCF-10A*erb*B-2 cell population did form some small colonies. However, MCF-10A*erb*B-2 cells formed colonies only at a very low efficiency and with small diameters compared to the MCF-10A*erb*B-2shH cells. This indicates that the quantitative difference in c-*erb*B-2 gene expression between these different lines (i.e. moderate compared to high levels of $p185^{erbB-2}$) significantly determined the threshold level of signaling required for the effective transformation of human mammary epithelial cells in culture. *In vivo* experiments have also been carried out using nude mice as recipients. As previously reported, MCF-10A and MCF-10A*erb*B-2 cells never formed tumors in nude mice, however, MCF-10A*erb*B-2shH cells did form small tumors in nude mice. Early experiments utilizing direct s.c. injection of cell suspension required over 10^7 cells for tumor formation, but more recent injections in which the cells were embedded in Matrigel matrix showed better tumor formation using lower numbers of cells. Thus, while the original MCF-10A*erb*B-2 cells did not display a significantly transformed phenotype in culture or *in vivo*, MCF-10A*erb*B-2shH cells form foci, grow efficiently under anchorage-independent conditions in soft agarose culture and form tumors in nude mice. This indicates that the high-level of c-*erb*B-2 gene overexpression seen throughout the MCF-10A*erb*B-2shH cell population induces a fully transformed phenotype.

In one of the previous studies mentioned above, it was also reported that the growth of heterogenous c-*erb*B-2-overexpressing transfected cell populations in nude mice showed much greater elevation of c-*erb*B-2 gene expression in tumor cells which grew out *in vivo* then did the original transfectants, and this higher level of c-*erb*B-2 overexpression correlated with increased soft agarose growth in culture and increased tumor growth *in vivo*. This, when combined with our own correlative data where we reported progressive growth factor independence associated with increased *erb*B-2/*erb*B-3 and PI 3-kinase activation in the 21T breast cancer cell lines, had suggested to us that the selection of variants containing higher levels of c-*erb*B-2 overexpression may be required to induce full neoplastic transformation. In a sense, this is also similar to that what had already been reported in very early studies using NIH3T3 cells, where the ability of wild-type c-*erb*B-2 to transform NIH3T3 cells was only seen in the cell lines that were engineered to overexpress very high levels of c-*erb*B-2 (approximately greater than 7×10^8 receptors per cell). However, much question is often raised about the relevance of such studies in NIH3T3 cells. But our present data now further supports this contention for mammary epithelial cells as well, and this may help clarify some of the confusion in the literature, where the potential quantitative differences in c-*erb*B-2 gene overexpression in the different genetically engineered mammary epithelial cell lines was never thoroughly investigated or emphasized.

In order to accomplish objectives 2 and 4 of the grant, we have also focused on specific strategies for blocking $p185^{erbB-2}$/*erb*B-3 function in cells stimulated with HRG as well as in breast cancer cells with c-*erb*B-2 gene amplification. The relative importance of $p185^{erbB-2}$/*erb*B-3 function (relative to $p185^{erbB-2}/p185^{erbB-2}$ homodimer function) in the constitutive activation of PI 3-kinase and the transformation of tumor cells with c-*erb*B-2 gene amplification has not yet been clearly established. One strategy which has been used successfully for other receptor tyrosine kinases employs dominant negative vectors, wherein the region coding for the cytoplasmic region of the receptor is almost completely removed. While the mutant truncated receptor still contains the transmembrane domain and can thus dimerize within the cell, it lacks tyrosine kinase activity and inhibits the signal transduction docking function of both the wild-type and mutant receptors within heterodimers. Therefore, by specifically removing this region the receptor function is completely impaired. This strategy has been used effectively for blocking EGFR, platelet-derived growth factor receptor, fibroblast growth factor receptor, and mutated *neu* function in various studies. In order to block the HRG-induced activation of $p185^{erbB-2}$/*erb*B-3 as well as the constitutive activation of $p185^{erbB-2}$/*erb*B-3 in breast cancer cells, we have constructed expression vectors that code for dominant negative forms of $p185^{erbB-2}$ and *erb*B-3 for introduction into

various cell lines. Constructing the dominant negative vector for c-*erb*B-3 was an especially important focus for this project, because this has not been previously attempted and may be especially effective in blocking the heterodimer interactions between $p185^{erbB-2}$ and *erb*B-3.

As originally outlined in the project proposal, we attempted to clone various PCR-generated fragments of c-*erb*B-2 and c-*erb*B-3 cDNAs into the pSLH1001 bicistronic retroviral vector. While the amplification of the 2 kb regions of reverse-transcribed mRNAs was accomplished successfully using a high-fidelity PCR kit, these fragments could not be ligated into the pSLH1001 vector. More recently, we have successfully introduced PCR-generated fragments coding for different forms of c-*erb*B-2 and c-*erb*B-3 into pSLH1001 using alternate primers. However, we were not able to detect the expression of the dominant negative proteins in recipient cell lines using these previously constructed vectors (see previous progress reports). One serious obstacle to strategies for constructing expression vectors with PCR-generated fragments may involve the deleterious mutations that occur at a significant frequency in amplified products, even in protocols using high-fidelity Pou/Taq polymerase enzyme combinations. An alternate approach for developing the dominant negative expression vectors has now been employed using the full-length cDNAs. By using a standard plasmid expression vector as an intermediate, we have cloned both wild-type and dominant negative c-*erb*B-3 into the pSLH1001 bicistronic retroviral expression vector using flanking restriction sites located within the extensive polylinker region of a standard plasmid vector. Either the wild-type c-*erb*B-3 or the dominant negative 2.2 kb c-*erb*B-3 fragment lacking most of the cytoplasmic domain of the gene was ligated into the plasmid vectors. These ligations also introduced in frame stop codons downstream of the point of ligation. These vectors were then used directly in experiments in which they were transfected cells. However, for introduction of the vectors into various cell lines that do not show reasonably high transfection efficiencies with antibiotic resistance and marker gene co-expression when transfected with standard monocistronic vectors (see previous progress reports), the newly constructed bicistronic retroviral expression vectors are highly useful in that they show 100% co-expression of antibiotic resistance with marker gene expression, and thus eliminates the occurrence of false positive clones. The wild-type and dominant negative forms of c-*erb*B-3 have now been successfully cloned into the SLH1001 vector. Extensive restriction digest analysis has confirmed the proper construction of the vectors and the expression of the dominant negative *erb*B-3 protein in the infected cell lines was confirmed using immunocytochemistry with an anti-*erb*B-3 antibody that specifically binds the extracellular domain of *erb*B-3. These cells have now been used for further analysis of $p185^{erbB-2}$/*erb*B-3 function in normal and breast cancer cells.

Studies have been carried out using these cell lines to determine the effects of dominant negative *erb*B-3 expression on the activation of PI 3-kinase after stimulation with HRG in H16N-2 and 21MT-1 cells, or that is seen constitutively in the 21MT-1 cells. The expression of dominant negative *erb*B-3 was found to inhibit both the HRG-induced activation of PI 3-kinase in the H16N-2 and 21MT-1 cell lines, as well as the constitutive activation of PI 3-kinase in the 21MT-1 cells. Monolayer growth assays under serum-free conditions also showed that dominant negative *erb*B-3 expression inhibited the HRG-induced proliferation of both H16N-2 and 21MT-1 cells in culture. The low tumor forming efficiency of the higher passage 21MT-1 cells has been a problem for testing the effects of dominant negative *erb*B-3 *in vivo*. Work pertaining to other grants is continuing using these and other tumor cell lines in culture studies, as well as *in vivo* in nude mice. Future work pertaining to other grants will investigate the effects of the dominant negative *erb*B-3 vector on signaling and growth in these and additional cell lines.

APPENDICES

1) Key research accomplishments:

- Developed a series of cell lines from the MCF-10A*erb*B-2 cells which overexpress variously elevated levels of c-*erb*B-2 comparable to that in breast cancer cells.
- Used the new MCF-10A*erb*B-2-derived cell lines to study the growth factor independence, HRG responsiveness, anchorage-independent growth and the level of PI 3-kinase constitutively activated as a function of c-*erb*B-2 overexpression.
- Used the new MCF-10A*erb*B-2-derived cell lines to study the tumorigenic potential of these cell lines *in vivo* in nude mice.
- Developed a new set of cell lines that express a dominant negative form of c-*erb*B-3.
- Used these new cell lines to study the effects of dominant negative *erb*B-3 on the HRG responsiveness and the level of PI 3-kinase activation in culture.
- Used these new cell lines to study the effects of dominant negative *erb*B-3 on the tumorigenic potential of these cell lines *in vivo*.

2) One abstract was presented at the AACR meeting this last spring. This work was also presented in 2 invited talks given earlier this year. A manuscript including much of this work is presently in preparation.

3) A copy of the abstract presented at the AACR meeting is attached.

#4714 Apoptosis enhances Lewis Y dependent procoagulant activity. Adachi, T., Inufusa, H., Hashimoto, Y., Wakano, T., Krimoto, M., Shindo, K., and Yasutomi, M. *Dept. of 1st Surgery, Kinki University School of Med., Osakasayama 589-8511 Japan and Fujisaki Inst., Hayashibara Biochemical Labs. Inc., (KM) Okayama 702-8006 Japan.*

Procoagulant activity of cancer cells involves in their metastatic potential and causes disseminated intravascular coagulation (DIC) in cancer patients. Cancer cell-derived blood coagulating activity 1 (CCA-1) is a tumor procoagulant which activates the blood coagulation factor X, and the characteristics differ from 68 kDa cysteine protease, and Le^y glycolipid acts as an essential cofactor of CCA-1. Apoptosis of cancer cell enhances expression of Le^y antigen, which is a cofactor for cancer cell-derived blood-coagulating activity-1 (CCA-1). Human pancreas cancer cell line MIA-PaCa-2 and colon cancer cell line DLD-1 were treated with 5-aza-2′-deoxycytidine (ADC) that is an inhibitor of DNA methylation and induces cell differentiation for 7 days. The expression intensity of cell surface Le^y was examined with flow cytometry, apoptosis was calculated from sub-G1 peak as DNA fragmentation and Le^y dependent procoagulant activity was investigated using clot timer with anti-Le^y monoclonal antibody FS01 that specifically inhibits procoagulant activity of CCA-1. ADC enhanced DNA fragmentation, Le^y expression and CCA-1 activity of MIA-PaCa-2, and Le^y expression and CCA-1 activity of DLD-1. Apoptosis enhances cell surface expression of Le^y and Le^y dependent procoagulant activity also. These results suggests that apoptosis induced Le^y play an important roll in DIC of cancer patients.

#4715 Characterization of the squamous carcinoma of human uterine cervix by sulfolipids and differentiation-related markers in relation to its involvement in the cellular signaling. Kiguchi, K., Saga, M., Okamura, A., Iwamori, M., Amemiya, A., and Yasumoto, S. *Tokyo Hospital, St. Marianna Univ. School of Medicine, Kanagawa 211, Japan. Faculty of Medicine, Univ. of Tokyo, Tokyo 113, Japan. St. Marianna Univ. School of Medicine, Kanagawa 216, Japan. Kanagawa Cancer Research Center, Kanagawa 241, Japan.*

Cholesterol sulfate (CS) is known to modulate the activity of protein kinase (PKC), form η; that plays a critical part in the differentiation of squamous keratinocytes. To gain a new insight into the regulatory mechanism of cellular differentiation and tumorigenesis by CS, we examined the content of CS in freshly obtained human cervical cancer tissues, as well as in several cell lines established from the cervical cancers. Although CS contents were various among the uterine cervical cancers, higher levels of the CS were well-correlated with strong expression of cytokeratin in the tissues as revealed by the immunostaining. Similar correlations were also obtained in several cervical cancer cell lines. Since we have previously shown good correlations between the level of CS contents and the activity of Transglutaminase-1(TG-1), we further examined whether CS could have a potential for the activation of TG-1 in these cancer cells. By using cell free system, it was demonstrated that CS had strong activity to phospholylate TG-1 by the microsome fraction, but not cytosol. These results strengthen the notion that CS is a potent second messenger *via* membrane bound PKC η to activate TG-1 in the process of cellular differentiation of cervical cancer cells.

#4716 Systematic isolation and characterization of genes involved in the regulation of growth/differentiation of embryonic mouse skin by RNA differential display. Takaishi, M., Makino, T., Takata, Y., Yamago, G., and Huh, N. *Toyama Medical and Pharmaceutical University, Toyama, Japan.*

For the better understanding of normal growth and differentiation as well as malignant conversion of epidermal cells, it is critically important to systematically isolate and characteriza involved genes. For this purpose, we utilized characteristic developmental change of mouse epidermal architecture, i.e., the simple basal layer of epidermis at 13.5 dpc stratifies progressively by the addition of more differentiated layers, resulting in a fully differentiated cornified epithelium at 16.5 dpc. By RNA differential display using epidermal RNA from 12.5 dpc to 16.5 dpc, seven yet unknown genes were isolated. Among those characterized until now, one designated as 4C32 was deduced as a keratin-associated protein from the sequence homology and characteristic amino acid repeats. Another gene, 4A40, was a large transcript (>10 kb) and contained repetitive amino acid sequences with spacer, possibly processed proteolytically in a similar way as profilaggrin. A gene designated as 4A50 showed sequence homology with human infertility-related 75 kDa protein, and its expression level actually increased with the maturation of testis. The present results indicate that our approach is suitable to systematically isolate genes involved in growth/differentiation of skin, and that characterization of the isolated genes will provide intriguing information on skin biology and oncology.

#4717 Critical threshold level of c-*erb*B-2 gene overexpression required for full neoplastic transformation of MCF-10A human mammary epithelial cells. Ram, T.G., Hosick, H.L., and Ethier, S.P. *Washington State University, Pullman, WA 99164 and University of Michigan Medical Center, Ann Arbor, MI 48109.*

Much recent evidence suggests that a high *critical threshold* level of wild-type c-*erb*B-2 gene overexpression is required to effectively transform human mammary epithelial cells. We are particularly interested in how high the level of c-*erb*B-2 gene expression must be to constitutively activate key signal transduction pathways that fully transform human mammary epithelial cells, and how the cooperative effects of $p185^{erbB\text{-}2}$/*erb*B-3 heterodimer action affects this critical threshold. We have now successfully produced cell populations derived from the immortalized MCF-10A non-neoplastic human mammary epithelial cell line that overexpress c-*erb*B-2 at very high levels truly comparable to that seen in breast carcinoma cells with c-*erb*B-2 gene amplification. While the original clones of MCF-10AerbB-2 cells overexpress c-*erb*B-2 at only moderate levels and are not tumorigenic in nude mice, MCF-10AerbB-2 cells selected for high-level $p185^{erbB\text{-}2}$ overexpression and growth factor independence in culture show a high level of constitutive phosphatidylinositol (PI) 3-kinase activity and a fully transformed phenotype both in culture and *in vivo*. In addition, key observations were made during the course of these studies concerning cell survival, proliferation in the absence of growth factors, heregulin responsiveness, and anchorage-independent growth that occurs as a function of high-level $p185^{erbB\text{-}2}$ overexpression. Experiments are continuing to study the changes in the constitutive activation of $p185^{erbB\text{-}2}$ and *erb*B-3 in these cell lines, and to relate the accompanying changes in PI 3-kinase activation (and other effects of $p185^{erbB\text{-}2}$ overexpression) to the threshold level of signaling required for effective transformation.

#4718 Redox regulation of the ras pathway. Tammy B. DeHahn, Magda C. Gutowski, Katherine A. Shackelford, Pamela G. Rutherford, Julia K. Enkema, Dianna L. Bailey, David A. Johnson, and Warren C. MacKellar. *Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285.*

Mutations in Ki-ras are found in over 30% of human tumors. These mutations lead to a constituitively active ras by trapping the ras protein in the GTP-bound form (activated ras). GTP binding to H-ras has been shown to be stimulated by reactive oxygen species (ROS) and free radical compounds such as nitric oxide. H-ras transformed cells also produce high levels of ROS. However, the effects of ROS on Ki-ras have not been investigated. In this study hemin, a generator of superoxide, stimulates GTP binding to Val-12 Ki-ras, while H_2O_2 inhibits binding of GTP to K-ras. Other redox compounds have been shown to both inhibit and stimulate ras-GTP binding depending upon buffer conditions. The mechanism of this duality will be discussed. Initial studies utilizing N-acetyl cysteine to modulate the redox state of the cell have demonstrated a decrease in phosphorylation of MAP kinase, a downstream component of the Ras pathway. This redox modulation may be important in both transformation and cellular proliferation.

#4719 Expression of the BOG (RBBP-9) gene during activation and expansion of hepatic stem cells. Ashida, K., Woitach, J.T., Loi, R., and Thorgeirsson, S.S. *Laboratory of Experimental Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD 20892.*

Bog is a pRb binding protein, whose over-expression in normal rat liver epithelial (RLE) cells conferred resistence to the growth inhibitory effect of TGF-β1 and ultimately leads to a transformed phenotype in vitro. We investigated the expression and cellular localization of BOG transcripts in two models of liver regeneration, partial hepatectomy (PH) alone and 2-acetylaminofluorene (AAF)/PH model. In both models BOG expression increases during liver regeneration. Prior to PH, BOG transcripts were observed in bile epithelial cells and sinusoid lining cells, but not detected in hepatocytes. After PH, expression of BOG is seen also in hepatocytes. In the AAF/PH model, characterized by selective proliferation of oval and stellate cells, increased levels of BOG transcripts are primarily observed in oval and stellate cells with low expression seen in the non-proliferating hepatocytes. The increased expression of BOG was coincidental with high level of TGF-β1 expression observed in the AAF/PH model. We propose that a transient increase in BOG expression in vivo may facilitate expansion of the early progeny of hepatic stem cells.

#4720 Nuclear localization of the BOG (RBBP-9) protein is dependent on pRb binding motif. Loi, R., Woitach, J.T., Factor, V., Ashida, K., and Thorgeirsson, S.S. *Laboratory of Experimental Carcinogenesis, NCI, NIH, Bethesda, MD 20892.*

We have recently identified BOG (RBBP-9), a novel pRb binding protein (Nature Genetics **19:** 371–374, 1998). BOG interacts with pRb, p107 and p130 through the LXCXE motif. Substitution of a leucine with glutamine in the LXCXE sequence (BOGΔQ) is sufficient to disrupt the interaction between BOG and pRb, p107 and p130. Using immunohistochemistry we demonstrate that BOG is primarily located in the nuclei of RLE cells and several mouse tissues. We have confirmed the nuclear localization of the BOG protein by transiently transfecting cells with the recombinant vector expressing the fusion protein BOG-EGFP (Enhanced Green Fluorescent Protein). When BOGΔQ-EGFP is expressed, cytoplasmic localization predominates, demonstrating that the integrity of the pRb binding motif is necessary for the nuclear localization of the BOG protein. Saos-2 cells which express a functionally inactive form of pRb were transfected with the BOG-EGFP vector and despite the presence of primarily cytoplasmic pRb, BOG was still localized to the nucleus, suggesting that the binding with the pRb related proteins p107 and/or p130 may support translocation of the fusion protein to the nucleus in the absence of functional pRb.

#4721 Overexpression of Phospholipase D in human breast cancer tissues. Noh D.Y.[1], Huh S.K.[1], Ahn S.J.[1], Suh, P.G.[2], Ryu S.H.[2], and Han J.S.[3] *[1]Seoul National University College of Medicine, Seoul, [2]Pohang Institute of Technology, Pohang, Korea, [3]Hanyang University College of Medicine, Seoul.*